

Report on the Verification of the Performance of MON 531 and MON1445 Event-specific Methods on the Cotton Event MON 531 x MON 1445 Using Real-time PCR

Validation Report and Protocols

C. Savini, D. Charels, A. Bogni, M. Mazzara, G. Van den Eede



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European Commission
Joint Research Centre
Institute for Health and Consumer Protection

Contact information

Address: Molecular Biology and Genomics Unit

E-mail: JRC-BGMO@ec.europa.eu

Tel.: +39 0332 789379

Fax: +39 0332 786159

<http://ihcp.jrc.ec.europa.eu/>

<http://www.jrc.ec.europa.eu/>

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6 March 2009

**Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Executive Summary

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) which combines the MON 531 and MON 1445 transformation events. The two methods have been previously validated individually on single-trait events, to detect and quantify each event in cotton samples; a validation report for each method is available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>. This study was conducted according to internationally accepted guidelines ^(1, 2).

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Europe S.A. provided the detection methods and the control samples: whole seeds of MON 531 x MON 1445 cotton (ST5599BR) and whole conventional cotton seeds of ST474. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.


Drafted by:

D. Charels



Report Verification Team:

1) L. Bonfini



2) G. Pinski



Scientific and technical approval:

M. Mazzara



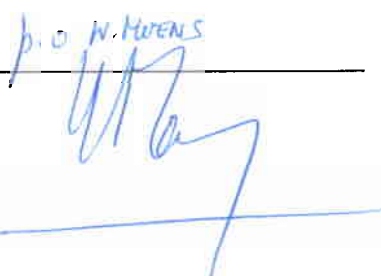
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S. Cordeil



Authorisation to publish:

G. Van den Eede



Address of contact laboratory:

European Commission, Joint Research Centre (JRC)

Institute for Health and Consumer Protection (IHCP)

Molecular Biology and Genomics Unit – Community Reference Laboratory for Genetically
Modified Food and Feed

Via Fermi 2749, I-21027 Ispra (VA)

Italy

Report on Steps 1-3 of the Validation Process

Monsanto Europe S.A. submitted the detection methods and control samples of the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Community Reference Laboratory for GM Food and Feed (CRL-GMFF), following reception of the documentation and material, including control samples, (step 1 of the validation process) carried out the scientific assessment of documentation and data (step 2) in accordance with Commission Regulation (EC) No 641/2004 "on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation" and according to its operational procedures ("Description of the CRL-GMFF Validation Process", <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

The scientific assessment focused on the method performance characteristics assessed against the method acceptance criteria set out by the European Network of GMO Laboratories and listed in the "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/doc/Method%20requirements.pdf>) (see Annex 1 for a summary of method acceptance criteria and method performance requirements). During step 2, five scientific assessments were performed and three requests of complementary information were addressed to the applicant. Upon reception of the complementary information, the scientific assessment of the detection method for the MON 531 x MON 1445 cotton was concluded in May 2006.

The event-specific detection methods for the two cotton lines hosting the single events MON 531 and MON 1445 were validated by the CRL-GMFF following the conclusion of the respective international collaborative studies and the publication of the validation reports (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). Hence, the detection methods applied on the cotton event MON 531 x MON 1445 did not undergo a full validation process. The CRL-GMFF performed an in-house verification of the detection methods to verify that they exhibit a comparable performance on samples of event MON 531 x MON 1445 combining the two traits (as provided in accordance to Annex 1.2.C.2 of Commission Regulation (EC) No 641/2004).

In October 2006, the CRL-GMFF concluded the experimental verification of the method characteristics (step 3, experimental testing of the samples and methods) by quantifying, with each specific method, five blind GM-levels within the range 0.1 %-6 %, on a DNA/DNA ratio. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, trueness and repeatability of the quantification were within the limits established by the ENGL.

A Technical Report summarising the results of tests carried out by the CRL-GMFF (step 3) is available on request.

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1. Introduction

The applicant Monsanto Europe S.A. submitted the detection methods for MON 531 and MON 1445 and the control samples of the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) under Article 5 and 17 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council "on genetically modified food and feed".

The Joint Research Centre (JRC, Molecular Biology and Genomics Unit of the Institute of Health and Consumer Protection) as Community Reference Laboratory for GM Food and Feed, established by Regulation (EC) 1829/2003, carried out an in-house verification of the two event-specific methods for the detection and quantification of MON 531 and MON 1445 in the MON 531 x MON 1445 cotton event combining the two traits. The single methods had been previously validated by international collaborative studies on the single-trait cotton events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

Upon reception of methods, samples and related data (step 1), the CRL-GMFF carried out the assessment of the documentation (step 2) and the in-house evaluation of the methods (step 3) according to the requirements of Regulation (EC) 641/2004 and following CRL-GMFF operational procedures. The CRL-GMFF method verification was concluded in October 2006.

A method for DNA extraction from cotton seeds, submitted by the applicant, was evaluated by the CRL-GMFF in order to confirm its performance characteristics. The protocols for DNA extraction are available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

The operational procedure of the in-house verification included the following module:

- ✓ Quantitative real-time PCR (Polymerase Chain Reaction). The methodology consists of two event-specific real-time quantitative TaqMan[®] PCR procedures for the determination of the relative content of events MON 531 and MON 1445 DNA to total cotton DNA in the MON 531 x MON 1445 cotton event. The procedures are simplex systems, in which the events MON 531 and MON 1445 were quantified in reference to the cotton *acp1* (acyl carrier protein) endogenous gene.

The study was carried out in accordance to the following internationally accepted guidelines:

- ✓ The IUPAC "Protocol for the design, conduct and interpretation of method performance studies" ⁽¹⁾.
- ✓ ISO 5725:1994 ⁽²⁾.

2. Materials

For the verification of the quantitative event-specific methods, control samples consisting of:

- genomic DNA extracted from homogenised seeds of MON 531 x MON 1445 cotton (ST5599BR),
- genomic DNA extracted from homogenised seeds of conventional cotton ST474,

were provided by the applicant, in accordance to the provisions of Regulation (EC) No 1829/2003, Art 2.11 ["control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample)"].

Samples containing mixtures of 100 % MON 531 x MON 1445 and conventional cotton genomic DNA at different GMO concentrations were prepared by the CRL-GMFF in a constant amount of total cotton DNA, using the control samples provided.

The protocols (reagents, concentrations, primer/probe sequences) followed in the in-house verification are those already published as validated methods for the individual MON 531 and MON 1445 events and available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

Table 1 shows the five GM levels of blind samples used in the verification of the MON 531 and MON 1445 methods.

Table 1. MON 531 and MON 1445 GM contents in cotton event MON 531 x MON 1445

MON 531 GM% (GM DNA / Non-GM DNA x 100)	MON 1445 GM% (GM DNA / Non-GM DNA x 100)
0.10	0.10
0.50	0.50
0.90	0.90
2.50	2.50
6.00	6.00

3. Experimental design

Eight runs for each event-specific method were carried out. In each run, samples were analysed in parallel with both the GM-specific system and the reference system (*acp1*). Five GM levels per run were examined and two replicates for each GM level were analysed. PCR analysis was performed in triplicate for all samples. In total, for each method (MON 531 and MON 1445), the quantification of the five GM levels was performed as an average of sixteen replicates per GM level. An Excel spreadsheet was used for determination of GM%.

4. Method

Description of the operational steps

For specific detection of events MON 531 and MON 1445 in cotton event MON 531 x MON 1445, two specific fragments of the integration regions of the constructs inserted into the plant genome, of 72 bp and 87 bp respectively, are amplified using specific primers.

PCR products are measured during each cycle (real-time) by means of target-specific oligonucleotide probes labelled with two fluorescent dyes: 6-FAM is used for MON 531 and MON 1445 as reporter dye at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of events MON 531 and MON 1445 DNA, a cotton-specific reference system which amplifies a 76 bp fragment of the cotton endogenous gene *acp1* (acyl carrier protein), using two *acp1* gene-specific primers and an *acp1* gene-specific probe labelled with FAM and TAMRA, was used.

Standard curves are generated for each GM specific system (MON 531 or MON 1445), by plotting Ct values of the calibration standards against the logarithm of the DNA copy numbers MON 531 or MON 1445 DNA, and fitting a linear regression into these data. Thereafter, the Ct values of the blind samples are measured and, by means of the regression formula, the relative amount of event MON 531 or MON 1445 DNA is estimated respectively.

For detailed information on the preparation of the standard curve calibration samples please refer to the protocols of the validated methods at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

5. Deviations reported

No deviations were introduced.

6. Summary of results

PCR efficiency and linearity

The values of the slopes of the standard curves, from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})}-1] \times 100$, and of the R^2 (expressing the linearity of the regression) reported for all PCR systems in the eight runs, are presented in Table 2 and 3 for MON 531 and MON 1445 methods, respectively.

Table 2. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 531 method on event MON 531 x MON 1445.

Run	MON 531			acp1		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.22	104	0.99	-3.46	94	1.00
2	-3.22	104	0.99	-3.46	94	1.00
3	-3.12	109	1.00	-3.38	98	1.00
4	-3.12	109	1.00	-3.38	98	1.00
5	-3.21	105	1.00	-3.42	96	1.00
6	-3.21	105	1.00	-3.42	96	1.00
7	-3.25	103	1.00	-3.41	97	1.00
8	-3.25	103	1.00	-3.41	97	1.00
Mean	-3.20	105	1.00	-3.42	96	1.00

Table 3. Values of standard curve slope, PCR efficiency and linearity (R^2) for the MON 1445 method on event MON 531 x MON 1445.

Run	MON 1445			acp1		
	Slope	PCR Efficiency (%)	Linearity (R^2)	Slope	PCR Efficiency (%)	Linearity (R^2)
1	-3.28	102	0.98	-3.38	98	0.99
2	-3.32	100	0.98	-3.30	101	0.99
3	-3.19	106	0.99	-3.28	102	0.99
4	-3.06	112	0.99	-3.20	105	0.99
5	-3.29	101	0.99	-3.19	106	0.99
6	-3.33	100	0.99	-3.23	104	0.99
7	-3.21	105	0.99	-3.24	103	0.99
8	-3.14	108	0.98	-3.19	106	0.99
Mean	-3.23	104	0.99	-3.25	103	0.99

The mean PCR efficiencies of the GM specific systems are 105 % and 104 % respectively for the MON 531 and MON 1445 specific systems. The linearity of the methods is equal to or

above 0.98 for both systems. Overall, data reported in Table 2 and 3 confirm the appropriate performance characteristics of the two methods tested on MON 531 x MON 1445 cotton samples in terms of PCR efficiency and linearity.

7. Method performance requirements

The results of the in-house verification study for the MON 531 and MON 1445 detection methods applied to event MON 531 x MON 1445 cotton DNA are reported in Tables 4 and 5, respectively. Results were evaluated with respect to the method acceptance criteria, as established by ENGL and adopted by the CRL-GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>, see also Annex 1). In addition, Tables 4 and 5 report estimates of the trueness and repeatability standard deviation for each GM level and for both methods.

Table 4. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation (RSD_r %) of the MON 531 method on event MON 531 x MON 1445 cotton DNA.

MON 531					
Blind sample GM%	Expected value (GMO%)				
	0.1	0.5	0.9	2.5	6
Mean	0.12	0.57	0.89	2.3	5.44
SD	0.02	0.05	0.05	0.21	0.65
RSD _r (%)	14	8.3	5.2	9.3	12
Bias (%)	23	15	-1.2	-8.1	-9.4

Table 5. Estimates of trueness (expressed as bias %) and relative repeatability standard deviation of the MON 1445 method on event MON 531 x MON 1445 cotton DNA

MON 1445					
Blind sample GM%	Expected value (GMO%)				
	0.1	0.5	0.9	2.5	6
Mean	0.08	0.42	0.9	2.47	6.29
SD	0.01	0.05	0.08	0.29	0.60
RSD _r (%)	9.6	13	8.6	12	9.5
Bias (%)	-16	-17	0.5	-1.1	4.8

The *trueness* of the method is estimated using the measures of the method bias for each GM level. According to the ENGL acceptance criteria and method performance requirements, the trueness of the method, measured as bias from the accepted value, should be within $\pm 25\%$ across the entire dynamic range. As shown in Tables 4 and 5, both methods satisfy the above requirement throughout their respective dynamic ranges.

Tables 4 and 5 further document the relative repeatability standard deviation (RSD_r) as estimated for each GM level. In order to accept methods for collaborative trial evaluation, the

CRL-GMFF requires that RSD_r values are below 25%, as indicated by ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>])). As it can be observed from the values reported in Tables 4 and 5, the two methods satisfy this requirement throughout their respective dynamic ranges.

8. Comparison of method performance between event MON 531 x MON 1445 and the single trait events

A synoptic comparison of the two method performances on the cotton event MON 531 x MON 1445 and the single trait events is shown in Tables 6 and 7. The performance of the methods on the single lines was previously assessed through international collaborative trials.

Table 6. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 531 detection method on event MON 531 x MON 1445 and on event MON 531.

Trueness and repeatability of MON 531 quantification on MON 531 x MON 1445			Trueness and repeatability of MON 531 quantification on single event MON 531*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.1	23	14	0.1	-22	34
0.5	15	8.3	0.5	-28	22
0.9	-1.2	5.2	0.9	-22	21
2.5	-8.1	9.3	2.5	-6.4	15
6	-9.4	12	6	2.5	21

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

Table 7. Trueness (bias %) and relative repeatability standard deviation (RSD_r %) of the MON 1445 detection method on event MON 531 x MON 1445 and on event MON 1445.

Trueness and repeatability of MON 1445 quantification on MON 531 x MON 1445			Trueness and repeatability of MON 1445 quantification on single event MON 1445*		
GM%	Bias (%)	RSD_r (%)	GM%	Bias (%)	RSD_r (%)
0.1	-16	9.6	0.1	41	14
0.5	-17	13	0.5	25	18
0.9	0.5	8.6	0.9	4.7	14
2.5	-1.1	12	2.5	11	11
6	4.8	9.5	6	5.2	17

*method validated (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>)

For trueness, the MON 531 even-specific method (Table 6), when applied to event MON 531 x MON 1445 and compared to the single line, shows a higher bias (%) at 6 % and a lower bias at GM levels 0.5 and 0.9 %. The MON 1445 event-specific method (Table 7), when applied to

event MON 531 x MON 1445, shows a lower bias (%) at all levels of GM. The trueness is within the acceptance value set by ENGL ($\pm 25\%$) for the dynamic range between 0.5 and 6.0% GM. However, the method for MON 1445 when applied to the single line event shows a bias of 41% at the 0.1 % GM level.

With the exception of the 2.5 % GM level of the MON 1445 method both the MON 531 and MON 1445 (Table 6 and 7) event-specific methods show lower values for relative repeatability standard deviation (RSD_r , %) when applied to the hybrid compared to the single events. With the exception of the 0.1 % GM level of the MON 531 event-specific method, the results are in all cases below the ENGL acceptance level established at maximum 25%.

Therefore, the in-house method verification has demonstrated that the MON 531 and MON 1445 detection methods developed to detect and quantify the single events can be equally applied for the quantification of the respective events combined in event MON 531 x MON 1445.

9. Conclusions

The overall method performance of the two event-specific methods for the quantitative detection of events MON 531 and MON 1445 combined in cotton event MON 531 x MON 1445 have been evaluated with respect to the method acceptance criteria and the method performance requirements recommended by the ENGL (as detailed under <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), and to the validation results obtained for the single trait events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results obtained during the present verification study indicate that the analytical modules of the methods submitted by the applicant comply with ENGL performance criteria. The methods are therefore applicable to the control samples provided (see section 3 "Materials"), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004.

10. Quality assurance

The CRL-GMFF carries out all operations according to ISO 9001:2000 (certificate number: CH-32231) and ISO 17025:2005 (certificate number: DAC-PL-0459-06-00) [DNA extraction, qualitative and quantitative PCR in the area of Biology (DNA extraction and PCR method validation for the detection and identification of GMOs in food and feed materials)].

11. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem*, 67: 331-343.
2. International Standard (ISO) 5725:1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization.

12. Annex 1: method acceptance criteria and method performance requirements as set by the European Network of GMO Laboratories (ENGL)

Method Acceptance Criteria should be fulfilled at the moment of submission of a method (Phase 1: acceptance for the collaborative study).

Method Performance Requirements should be fulfilled in a collaborative study in order to consider the method as fit for its purpose (Phase 2: evaluation of the collaborative study results).

Method Acceptance Criteria

Applicability

Definition: The description of analytes, matrices, and concentrations to which a method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. Euro/sample) of the method.

Acceptance Criterion: The practicability statement should provide indication on the required equipment for the application of the method with regards to the analysis *per se* and the sample preparation. An indication of costs, timing, practical difficulties and any other factor that could be of importance for the operators should be indicated.

Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance Criterion: The method should be event-specific and be functional only with the GMO or GM based product for which it was developed. This should be demonstrated by empirical results from testing the method with non-target transgenic events and non-transgenic material. This testing should include closely related events and cases where the limit of the detection is tested.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of accuracy and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below. The range of the standard curve(s) should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) end.

Accuracy

Definition: The closeness of agreement between a test result and the accepted reference value.

Acceptance Criterion: The accuracy should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of -3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation: Efficiency = $[10^{(-1/\text{slope})} - 1]$

Acceptance Criterion: The average value of the slope of the standard curve should be in the range of $(-3.1 \geq \text{slope} \geq -3.6)$

R² Coefficient

Definition: The R² coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R² should be ≥ 0.98 .

Repeatability Standard Deviation (RSD_r)

Definition: The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance Criterion: The relative repeatability standard deviation should be below 25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3 (1994).

Limit of Quantitation (LOQ)

Definition: The limit of quantitation is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an RSD_r $\leq 25\%$. Target concentration should be intended as the threshold relevant for legislative requirements. The acceptable level of accuracy and precision are described below.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single laboratory validation.

Acceptance Criterion: LOD should be less than $1/20^{\text{th}}$ of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring $\leq 5\%$ false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance Criterion: The response of an assay with respect to these small variations should not deviate more than $\pm 30\%$. Examples of factors that a robustness test could address are: use of different instrument type, operator, brand of reagents, concentration of reagents, and temperature of reaction.

Method Performance Requirements***Dynamic Range***

Definition: In the collaborative trial the dynamic range is the range of concentrations over which the reproducibility and the trueness of the method are evaluated with respect to the requirements specified below.

Acceptance Criterion: The dynamic range of the method should include the $1/10$ and at least five times the target concentration. Target concentration should be intended as the threshold relevant for legislative requirements.

Reproducibility Standard Deviation (RSD_R)

Definition: The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation should be below 35% at the target concentration and over the entire dynamic range. An $RSD_R < 50\%$ is acceptable for concentrations below 0.2%.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within $\pm 25\%$ of the accepted reference value over the whole dynamic range.



Event-specific Method for the Quantification of Cotton Line MON 531 Using Real-time PCR

Protocol

10 June 2008

**Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit**


Method development:

Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by
C. Delobel



Report Verification Team
1) M. Querci



2) M. Ermolli



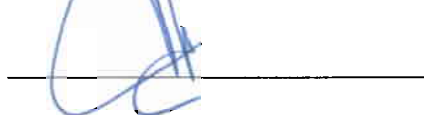
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M. Mazzara



Compliance to CRL Quality System
S. Cordeil



Authorisation to publish
G. Van den Eede



Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

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1. General information and summary of the methodology

This protocol describes a real-time quantitative TaqMan[®] PCR procedure for the determination of the relative content of event MON 531 DNA to total cotton DNA in a sample.

The PCR assay was optimised for the use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to the use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of event MON 531 genomic DNA, a 72-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' plant DNA region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM as a reporter at its 5' end and TAMRA as a quencher dye at its 3' end.

For relative quantification of event MON 531 DNA, a cotton-specific reference system amplifies a 76-bp fragment of a cotton fibre-specific *acy1* carrier protein gene (*acp1*), using two specific primers and one probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON 531 DNA in a test sample, MON 531 and *acp1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of event MON 531 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for DNA extracted from cotton seeds or grains containing mixtures of genetically modified and conventional cotton.

The reproducibility and trueness of the method was tested through an international collaborative trial using samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative study by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in July-August 2006.

Each participant received twenty blind samples containing cotton MON 531 genomic DNA at five GM contents, ranging from 0.1% to 6.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM cotton MON 531 in four unknown samples. Four replicates of each GM level were analysed on the same plate.

A detailed validation report can be found at <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.05% in 200 ng of total cotton DNA. The relative LOD was not assessed in the collaborative trial.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.1% in 200 ng of total cotton DNA. The lowest relative GM content included in the collaborative trial was 0.10%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to cotton event MON 531 and thus imparts event-specificity to the method.

The specificity of the assay was experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of MON 531, Roundup Ready[®] soybean 40-3-2, Roundup Ready[®] canola (RT200), Roundup Ready[®] canola (RT73), Roundup Ready[®] corn (GA21), Roundup Ready[®] corn (NK603), YieldGard[®] corn borer corn (MON 810), YieldGard[®] rootworm corn (MON 863), conventional corn, Bollgard[®] II cotton (MON 15985), conventional cotton, conventional Soybean, Roundup Ready[®] wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, Teosinte, hard wheat.

According to the applicant, none of the plant materials tested, except the positive control cotton line MON 531, Assoria rice and cotton MON 15985 gave detectable amplifications; MON 15985 however contains event MON 531 and was expected to be positive in PCR.

Assoria rice reacted unexpectedly with the event-specific detection assay of MON 531, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for MON 531 and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

The specificity of the cotton reference assay *acp1* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready® soybean 40-3-2, Roundup Ready® canola (RT200), Roundup Ready® canola (RT73), conventional canola, Roundup Ready® corn (GA21), Roundup Ready® corn (NK603), YieldGard® corn borer corn (MON 810), YieldGard® rootworm corn (MON 863), conventional corn, Roundup Ready® cotton (MON 1445), Bollgard® cotton (MON 531), Bollgard II® cotton (MON 15985), conventional cotton, conventional soybean, Roundup Ready® wheat (MON 71800), conventional wheat, Assoria rice, barley, basmati rice, Teosinte, lentil, quinoa, sunflower, oat, buckwheat, pinenuts, rye berries, millet, peanut.

According to the applicant, none of the plant materials tested, except cotton MON 531, cotton MON 15985, cotton MON 1445, conventional cotton and Assoria rice yielded detectable amplifications. Assoria rice reacted unexpectedly with the *acp1* assay, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for *acp1* and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2005.
- PCR-reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.

- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event MON 531

3.2.1 General

The PCR set-up for the taxon specific target sequence (*acp1*) and for the GMO (MON 531) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 531 in non-GM cotton DNA for a total of 200 ng of DNA (corresponding to approximately 85830 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA)⁽¹⁾.

A calibration curve is produced by plotting the Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software. The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

The ratio of GM copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents at 1-4 °C on ice.
2. In two reaction tubes (one for the MON 531 system and one for the *acp1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 531 specific system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 531 primer forward (10 µM)	150 nM	0.75
MON 531 primer reverse (10 µM)	150 nM	0.75
MON 531 probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *acp1* reference system.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
<i>acp 1</i> primer forward (10 µM)	150 nM	0.75
<i>acp 1</i> primer reverse (10 µM)	150 nM	0.75
<i>acp 1</i> probe (5 µM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

3. Mix gently and centrifuge briefly.
4. Prepare two reaction tubes (one for the MON 531 and one for the *acp 1* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).

5. Add to each reaction tube the correct amount of master mix (e.g. $46 \times 3 = 138 \mu\text{L}$ master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. $4 \times 3 = 12 \mu\text{L}$ DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
6. Spin down the tubes in a microcentrifuge. Aliquot 50 μL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately $250 \times g$ for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with cycling conditions described in Tables 3 and 4:

Table 3. Cycling program for the MON 531 system

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
	UNG	50 °C	120	No	1
1	Initial denaturation	95 °C	600	No	1
2	Amplification	Denaturation	95 °C	No	45
		Annealing & Extension	55 °C	60	

Table 4. Cycling program for the reference *acp1* system.

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
	UNG	50 °C	120	No	1
1	Initial denaturation	95 °C	600	No	1
2	Amplification	Denaturation	95 °C	No	45
		Annealing & Extension	60 °C	60	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curve in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the

amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at $Ct = 25 - 3 = 22$).

c) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *acp 1* and the MON 531 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of event MON 531 DNA in the unknown sample, the MON 531 copy number is divided by the copy number of the cotton reference gene (*acp 1*) and multiplied by 100 to obtain the percentage value ($GM\% = \text{MON 531} / \text{acp 1} \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON 531 target sequence	
531-F forward primer	5' - TCC CAT TCG AGT TTC TCA CGT -3'
531-R reverse primer	5' - AAC CAA TGC CAC CCC ACT GA -3'
531-P probe	FAM 5' - TTG TCC CTC CAC TTC TTC TC -3' TAMRA
Reference gene <i>acp1</i> target sequence	
<i>acp 1</i> forward primer	5' - ATT GTG ATG GGA CTT GAG GAA GA -3'
<i>acp 1</i> reverse primer	5' - CTT GAA CAG TTG TGA TGG ATT GTG -3'
<i>acp 1</i> probe	FAM 5' - ATT GTC CTC TTC CAC CGT GAT TCC GAA -3' TAMRA

5. References

1. Arumuganathan, K., Earle, E.D., (1991). Nuclear content of some important plant species. *Plant Mol Biol Reporter* 9: 208-218.



Event-specific Method for the Quantification of Cotton Line MON 1445 Using Real-time PCR

Protocol

06 June 2008

**Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit**

Method development:

Monsanto Company

Collaborative trial:

Community Reference Laboratory for GM Food and Feed (CRL-GMFF)
Biotechnology & GMOs Unit

Drafted by
C. Savini



Report Verification Team
1) M. Querci



2) M. Ermolli



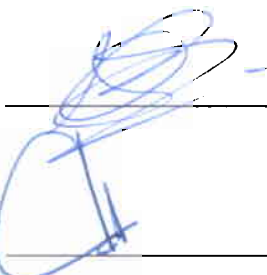
Scientific and technical approval
M. Mazzara



Compliance to CRL Quality System
S. Cordeil



Authorisation to publish
G. Van den Eede



Address of contact laboratory:

European Commission, Joint Research Centre
Institute for Health and Consumer Protection (IHCP)
Biotechnology and GMOs Unit – Community Reference Laboratory for GM Food and Feed
Via Fermi 2749, 21027 Ispra (VA) - Italy

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1. General information and summary of the methodology

This protocol describes a real-time quantitative TaqMan® PCR procedure for the determination of the relative content of cotton event MON 1445 DNA to total cotton DNA in a sample.

The PCR assay was optimised for use in real-time PCR instruments for plastic reaction vessels.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of cotton event MON 1445 DNA, an 87-bp fragment of the integration region of the construct inserted into the plant genome (located at the 5' plant DNA region) is amplified using two specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM as a reporter at its 5' end and TAMRA as a quencher dye at 3'-end.

For the relative quantification of cotton event MON 1445 DNA, a cotton-specific reference system amplifies a 76-bp fragment of the cotton gene *acyl carrier protein 1 (Acp 1)*, using a two specific primers and one probe labelled with FAM and TAMRA as described above.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of event MON 1445 DNA in a test sample, MON 1445 and *acp 1* Ct values are determined for the sample. Standard curves are then used to estimate the relative amount of cotton event MON 1445 DNA to total cotton DNA.

2. Validation status and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from cotton seeds or grains containing mixtures of genetically modified and conventional cotton.

The reproducibility and trueness of the method were assessed through an international collaborative ring trial using DNA samples at different GMO contents.

2.2 Collaborative trial

The method was validated in a collaborative study conducted by the Joint Research Centre (JRC) of the European Commission. The study was undertaken with twelve laboratories in June-July 2006.

Each participant received twenty blind samples containing cotton MON 1445 genomic DNA at five GM contents, ranging from 0.10% to 6.0%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each level of GM cotton MON1445 in four unknown samples. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.it/statusofdoss.htm>

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.04% in 200 ng of total cotton DNA. The relative LOD was not assessed in a collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.085% in 200 ng of total cotton DNA. The lowest relative GM content included in collaborative trial was 0.10 %.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region of recombination between the insert and the plant genome; the sequence is specific to cotton event MON1445 and thus imparts event-specificity to the method.

The specificity of the assay was experimentally tested by the applicant in real-time PCR against DNA extracted from plant materials containing the specific targets of MON 1445, Roundup Ready® soybean 40-3-2, Roundup Ready® Canola (RT200), Roundup Ready® Canola (RT73), Conventional Canola, Roundup Ready® Corn (GA21), Roundup Ready® Corn (NK603), YieldGard® Corn Borer Corn (MON810), YieldGard® Rootworm/Roundup Ready® Corn (MON88017), YieldGard® Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Bollgard® Cotton(MON 531), Bollgard® Cotton (MON757), BollgardII® Cotton (MON15985), Cotton MON88913, Conventional Cotton, Conventional Soybean, Roundup Ready® Wheat (MON71800), Conventional Wheat, Assoria rice, Barley, Basmati rice, Lentil, Quinoa, Sunflower Nuts, Oat, Buckwheat, Pinenuts, Rye berries, Millet, Peanut.

According to the applicant, none of the plant materials tested, except the positive control cotton line MON 1445 and Assoria rice, yielded detectable amplifications. Assoria rice reacted unexpectedly with the event-specific detection assay of MON 1445, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for MON 1445 and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

The specificity of the cotton reference assay *acp1* was experimentally tested by the applicant against DNA extracted from plant materials containing Roundup Ready[®] soybean 40-3-2, Roundup Ready[®] Canola (RT200), Roundup Ready[®] Canola (RT73), Conventional Canola, Roundup Ready[®] Corn (GA21), Roundup Ready[®] Corn (NK603), YieldGard[®] Corn Borer Corn (MON810), YieldGard[®] Rootworm/Roundup Ready[®] Corn (MON88017), YieldGard[®] Rootworm corn (MON863), Lysine Maize (LY038), Conventional Corn, Roundup Ready[®] Cotton (MON 1445), Bollgard[®] Cotton (MON 531), Bollgard[®] Cotton (MON 757), BollgardII[®] Cotton (MON 15985), Cotton MON 88913, Conventional Cotton, Conventional Soybean, Roundup Ready[®] Wheat (MON71800), Conventional Wheat, Assoria rice, Barley, Basmati rice, Toesinte, Lentil, Quinoa, Sunflower Nuts, Oat, Buckwheat, Peanuts, Rye berries, Millet, Peanuts.

According to the applicant, none of the plant materials tested, except Cotton MON 531, Cotton MON 757, Cotton MON 15985, Cotton MON 1445, Cotton MON 88913, conventional cotton and Assoria rice yielded detectable amplifications. Assoria rice reacted unexpectedly with the *acp1* assay, but this positive result is considered by the applicant to be an artefact. Bioinformatics analyses conducted by the CRL-GMFF confirmed the absence of relevant matches between the primers for *acp1* and the rice genome; this was also supported by additional tests conducted by the CRL-GMFF.

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. “forward flow direction” during PCR-setup, should follow international guidelines, e.g. ISO 24276:2005.
- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).

- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps - unless specified otherwise - should be carried out at 0 - 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of cotton event MON 1445

3.2.1 General

The PCR set-up for the taxon specific target sequence (*acp 1*) and for the GMO (event MON 1445) target sequence should be carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The use of maximum 200 ng of template DNA per reaction well is recommended.

The method is developed for a total volume of 50 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a 10% MON 1445 in non-GM cotton DNA for a total of 200 ng of DNA (corresponding to approximately 85830 cotton genome copies with one genome assumed to correspond to 2.33 pg of haploid cotton genomic DNA) ⁽¹⁾.

A calibration curve is produced by plotting the Ct-values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for the unknown sample DNA is obtained by interpolation from the standard curves.

The ratio of transgene copy number and reference gene copy number multiplied by 100 gives the % GM contents of the samples.

3.2.3 Real-time PCR set-up

1. Thaw, mix gently and centrifuge the required amount of components needed for the run.
Keep thawed reagents at 1-4°C on ice.
2. In two reaction tubes (one for the MON 1445 system and one for the *acp1* system) on ice, add the following components (Tables 1 and 2) in the order mentioned below (except DNA) to prepare the master mixes.

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the MON 1445 specific system.

Component	Final concentration	μL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
MON 1445 primer forward (10 μM)	150 nM	0.75
MON 1445 primer reverse (10 μM)	150 nM	0.75
MON 1445 probe (5 μM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the cotton *acp1* reference system.

Component	Final concentration	μL/reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
<i>acp 1</i> primer forward (10 μM)	150 nM	0.75
<i>acp 1</i> primer reverse (10 μM)	150 nM	0.75
<i>acp 1</i> probe (5 μM)	50 nM	0.50
Nuclease free water	#	19
Template DNA (max 200 ng)	#	4.0
Total reaction volume:		50

- Mix gently and centrifuge briefly.
- Prepare two reaction tubes (one for the MON 1445 and one for the *acp 1* master mixes) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
- Add to each reaction tube the correct amount of master mix (e.g. 46 x 3 = 138 μL master mix for three PCR repetitions). Add to each tube the correct amount of DNA (e.g. 4 x 3 = 12 μL DNA for three PCR repetitions). Vortex each tubes for approx. 10 sec. This step is mandatory to reduce the variability among the repetitions of each sample to a minimum.
- Spin down the tubes in a microcentrifuge. Aliquot 50 μL in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4 °C to room temperature) to spin down the reaction mixture.
- Place the plate into the instrument.
- Run the PCR with cycling conditions described in Table 3:

Table 3. Cycling program for the MON 1445 and *acp 1* systems

Step	Stage	T °C	Time (sec)	Acquisition	Cycles
1	UNG	50 °C	120	No	1
2	Initial denaturation	95 °C	600	No	1
3	Amplification	Denaturation	95 °C	No	45
		Annealing & Extension	60°C	60	

3.3 Data analysis

Subsequent to the real-time PCR, analyse the run following the procedure below:

a) Set the threshold: display the amplification curves of one system (e.g. MON 1445) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no “fork effect” between repetitions of the same sample. Press the “update” button to ensure changes affect Ct values. Switch to the linear view mode by clicking on the Y axis of the amplification plot, and check that the threshold previously set falls within the geometric phase of the curves.

b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at $Ct = 25 - 3 = 22$).

c) Save the settings.

d) Repeat the procedure described in a) and b) on the amplification plots of the other system (e.g. *acp 1* system).

e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *acp 1* and the MON 1445 specific systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the copy numbers in the unknown sample DNA.

For the determination of the amount of event MON1445 DNA in the unknown sample, the MON 1445 copy number is divided by the copy number of the cotton reference gene (*acp 1*) and multiplied by 100 to obtain the percentage value ($\text{GM\%} = \text{MON 1445}/\text{acp 1} * 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Vortex
- Rack for reaction tubes
- 1.5/2.0 ml reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4304437

4.3 Primers and Probes

Name	Oligonucleotide DNA Sequence (5' to 3')
MON1445 target sequence	
MON1445 primer forward	5' – GGAGTAAGACGATTCAGATCAAACAC – 3'
MON1445 primer reverse	5' – ATCGACCTGCAGCCCAAGCT – 3'
MON1445 probe	6-FAM 5' – ATCAGATTGTCGTTTCCCGCCTTCAGTTT – 3' TAMRA
Reference gene <i>acp 1</i> target sequence	
<i>acp 1</i> primer forward	5' – ATTGATGATGGGACTTGAGGAAGA – 3'
<i>acp 1</i> primer reverse	5' – CTTGAACAGTTGTGATGGATTGTG – 3'
<i>acp 1</i> probe	6-FAM 5' – ATTGTCCTCTCCACCGTGATTCCGAA – 3' TAMRA

European Commission

EUR 24158 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: Report on the Verification of the Performance of MON 531 and MON 1445 Event-specific Methods on the Cotton Event MON 531 x MON 1445 Using Real-Time PCR

Author(s): C. Savini, D. Charels, A. Bogni, M. Mazzara, G. Van den Eede

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Abstract

The JRC as Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established by Regulation (EC) No 1829/2003, has carried out an in-house verification study to assess the performance of two quantitative event-specific methods on the cotton event MON 531 x MON 1445 (unique identifier MON-ØØ531-6 x MON-Ø1445-2) which combines the MON 531 and MON 1445 transformation events. The two methods have been previously validated individually on single-trait events, to detect and quantify each event in cotton samples; a validation report for each method is available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>. This study was conducted according to internationally accepted guidelines ^(1, 2).

In accordance to Regulation (EC) No 1829/2003 of 22 September 2003 on genetically modified food and feed and to Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003, Monsanto Europe S.A. provided the detection methods and the control samples: whole seeds of MON 531 x MON 1445 cotton (ST5599BR) and whole conventional cotton seeds of ST474. The JRC prepared the in-house verification samples (calibration samples and blind samples at different GM percentages).

The results of the in-house verification study were evaluated with reference to ENGL method performance requirements (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) and to the validation results on the individual parental events (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>).

The results of this CRL-GMFF in-house verification studies are made publicly available at <http://gmo-crl.jrc.ec.europa.eu/>.

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